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(71) Applicant: MOLECULAR ENGINEERING ASSOCIATES, INC. [US/US]; 2126 7th Avenue S., Birmingham, AL 35233 (US).

(72) Inventors: COMPANS, Richard, W. ; Route 1, Box 406, Helena, AL 35080 (US). RAY, Ranjit ; 1600 9th Avenue S, Apt. 4E, Birmingham, AL 35205 (US).

(74) Agent: HAGAN, Patrick, J.; Dann, Dorfman, Herrell & Skillman, Three Mellon Bank Center, Suite 900, 15th Street and South Penn Square, Philadelphia, PA 19102-2440 (US).

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(54) Title: INTRANASAL IMMUNIZATION AGAINST VIRAL INFECTION USING VIRAL GLYCOPROTEIN SUBUNIT VACCINE

(57) Abstract

Method for immunizing against viral infection by administering intranasally an immunogenically effective amount of a viral envelope subunit vaccine comprising a glycoprotein complexed with a lipid.

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INTRANASAL IMMUNIZATION AGAINST VIRAL INFECTION USING VIRAL GLYCOPROTEIN SUBUNIT VACCINE

Background of the Invention:

Parainfluenza viruses are members of the paramyxovirus group, which also includes mumps and Newcastle disease viruses. Human parainfluenza type 3 (PI3; hemadsorption type 1) virus, which is probably the most common among the parainfluenza viruses, causes severe respiratory disease, particularly in children. Parainfluenza viruses type 1 and type 2 have similar epidemiological patterns and often cause croup in children between 1 and 4 years of age. Antigenic relationships have been reported among parainfluenza viruses types 1 - 4 and also between parainfluenza and mumps viruses, although limited information has been obtained about the protein components involved.

Attempts to vaccinate children against parainfluenza viral infection with formalin-inactivated virus have been reported in the past, but such preparations did not offer effective protection. The results of subsequent studies concerning immunization against paramyxoviruses tend to indicate that inactivation of the virus by chemical treatment probably destroys some of the important antigenic sites responsibe for induction of a protective immune response.

Immunization against respiratory tract pathogens has also been proposed using a modified live

virus. Administration of attenuated virus has been attempted intranasally as well as through more conventional routes e.g. subcutaneously, intraperitontally, intramuscularly or intravenously. The elicitation of an immune response through intranasal administration of attenuated virus cannot be considered unexpected in such cases, because the modified live virus of the vaccine is following the natural route of infection of the wild-type virus, creating immunity through a sub-clinical infection. The use of modified live virus to effect immunization entails certain risk, however, in that the avirulent but still active virus may revert to its virulent state after administration to the recipient.

It has previously been documented that envelope glycoproteins, HN and F, of paramyxoviruses are responsible for initiation and progress of the infection process. Studies have shown that antibodies to these glycoproteins are effective in preventing infection.

We previously reported the discovery that a new viral subunit vaccine derived from human parainfluenza type 3 virus envelope glycoproteins complexed with lipid is capable of inducing an antibody response which is far superior to that obtained with the previously used formalin-inactivated viral vaccine preparations. Ray et al., J. Infect. Dis., 152: 1219-30 (1985). Studies conducted using this new subunit vaccine have shown that a single subcutaneous immunization affords complete protection from challenge infection. Id. It was also found that the isolated viral glycoprotein subunit vaccine, composed of a glycoprotein-lipid complex vesicle, was easier to

prepare, as compared with subunit vaccines heretofore proposed. The latter are typically isolated in such a way as to be rendered lipid free. See, for example, U.S. Patents Nos. 4,344,935, or 4,356,169 and Morein et al., J. Gen. Virol, 64: 1557-69 (1983). That a glycoprotein lipid complex has shown such exceptional ability to confer immunity is considered quite suprising, as lipids are generally regarded as non-antigenic and thus their presenece in a vaccine composition would be thought to reduce its immunogenic effectiveness.

Our viral glycoprotein subunit vaccine, its method of preparation and method of use are the subject of copending U.S. patent application Serial No. _____, filed _____.

The preparation of our subunit vaccine is carried out in such a way that the antigenic sites essential for obtaining the desired antibody response are not chemically altered, with the result that antigenicity is not compromised. Further, our vaccine preparation is free of any viral genome and so avoids the risk of infection. Accordingly, our subunit vaccine offers distinct advantages over chemically inactivated virus and modified live virus vaccines. Insofar as is known, however, intranasal administration of a viral coat subunit vaccine has not been proposed heretofore as an effective means for affording protection against infection. Because subunit vaccines include none of the viral genome, neither clinical nor sub-clinical infection could result from administration of such a vaccine. Thus, the knowledge on which previous intranasal, modified live virus vaccines was predicated would not have suggested that a subunit vaccine such as ours, which is comprised of two

envelope glycoproteins and the lipid bi-layer from the virus, could induce immunity by intranasal administration.

Brief Description of the Invention

In accordance with the present invention, there is provided a method for the intranasal administration of a viral glycoprotein subunit vaccine which gives rise to a protective immune response in recipients of the vaccine. Both systemic and local antibody responses to the viral glycoprotein subunit vaccine are elicited after intranasal immunization. This result stands in sharp contrast to that obtained by subcutaneous immunization with the same antigen dosage, which markedly increases the systemic antibody response, but elicites only a moderate local response in the bronchial tract and thus produces only limited protection from infection.

Brief Description of the Drawings

Referring to the drawings herein,

FIG. 1 shows the elution profile of hamster serum protein bound to a column of immobilized jacalin, eluted with melibiose (0.1M).

FIG. 2 shows an immunoelectrophoroetic pattern resulting from the analysis of rabbit antiserum to hamster IgA.

FIG. 3 shows the results of immune precipitation of ^{35}S-methionine-labeled parainfluenza type 3 virus infected LLC-MK_2 cell lysate with bronchial lavages from different test animal groups.

FIG. 4 graphically represents the relative amounts of IgA class-specific antibodies to virus envelope glycoproteins appearing in bronchial lavages of control and immunized test animals after challenge with parainfluenza type 3 virus.

DETAILED DESCRIPTION OF THE INVENTION

Any lipid-containing virus which has an antigenic glycoprotein component consitutes suitable material for use in the method of the present invention. The lipid component of the glycoprotein-lipid complex is derived from the host cells in which the virus is produced. The lipids are incorporated into the viral envelope, along with the virus-specified proteins, during envelope assembly in the host cell. The manner in which the vaccine is prepared causes the glycoproteins and lipids to form descrete complexes or vesicles. Rather than being an undesirable component of the resulting glycoprotein subunit vaccine as would be expected, the associated lipids appear to enhance the immunogenicity of the preparation as a whole by acting as an adjuvant. The ability to form the antigenic lipid-glycoprotein vesicles is a function of the chemical nature of glycoproteins and lipids in general, and thus is not restricted to any specific type of glycoprotein or lipid.

Among the better characterized viral glycoproteins, which are generally recognized as being antigenic, are two types which are known generically as receptor-binding glycoproteins and fusion glycoproteins. These are defined by their function in

the process of host cell infection, and may be known by different specific names in different viruses. At least one, and frequently both, are present in such well known disease causing agents as paramyxoviruses, influenza viruses, respiratory syncytial viruses, rabies virus, herpes viruses and human immunodeficiency viruses, the latter including the etiologic agent of acquired immune deficiency syndrome (AIDS).

Particularly well-characterized are the receptor-binding-type and fusion-type glycoproteins possessed by all members of the paramyxovirus group. Included in the group are the parainfluenza viruses, measles virus, mumps virus, respiratory syncytial virus, Newcastle disease virus, and Sendai virus. In parainfluenza viruses, these glycoproteins are referred to as HN (72,000 daltons) and F_o (54,000 daltons and 20,000 daltons), respectively, and are believed to be responsible for attachment or hemagglutination and neuraminidase activities (HN) and for progress of infection (F) by the virus. Both of these glycoproteins are known to be highly antigenic, and thus are particularly favored for use in practicing the immunization method of the invention. As will be readily apparent, the diseases caused by certain members of the paramyxovirus group, especially parainfluenza, measles and mumps, are very widespread in humans, especially among children, and may be responsible for causing unusually harmful symptoms and/or side effects in afflicted individuals.

The F glycoprotein is known, at least in the case of parainfluenza, to be potentially separable into

subunits. For the purposes of the present description, any reference to an F glycoprotein is intended to refer to either the F glycoprotein as a whole, or its individual subunits, all of which may be detergent-solubilized.

Although the method of the invention is described and exemplified herein primarily with reference to subunit vaccine derived from the virus envelope of PI3 virus, the present method is considered to have significantly broader application. It is believed that viral glycoprotein subunit vaccine of the type described herein, when administered intranasally according to the method of this invention, will provide effective protection against a variety of viral infections including, but not limited to, those caused by the class of paramyxoviruses, influenza viruses, respiratory syncytial viruses, herpes viruses, human immunodeficiency viruses and rabies viruses.

The subunit vaccines used in practicing the present invention are readily prepared according to techniques well-known to those skilled in the art. The virus of interest is cultured in a suitable host-cell culture, purified to remove cellular debris and treated with a dialyzable detergent, such as cholate or octyl-D-glucoside to solubilize the desired envelope glycoprotein. It is important that the detergent used be easily dialyzable, to insure that only the detergent will be removed during further processing to the vaccine. After solubilization, the detergent-soluble portion of the virus is separated from the insoluble nucleocapsid by centrifugation, or other suitable means. The supernatent liquid is then dialyzed to produce complexes consisting of endogenous lipids and viral glycoproteins, which constitute the immuogenic

agent of the resultant vaccine. A detailed description of the preparation of such a viral glycoprotein subunit vaccine is provided in Ray et al., J. Infect Dis., 152, pp. 1219-30 (1985), the entire disclosure of which is incorporated by reference in the present application for patent, as if set forth herein in full.The viral glycoproteins may also be produced by genetic engineering (e.g. using recombinant DNA technology) or other techniques for purposes of the invention.

Further purification of the glycoproteins may be achieved by affinity chromotography. The procedure for preparing monoclonal antibodies to the HN and F glycoproteins of human parainfluenza type 3 virus and the process of using those antibodies in the isolation and purification of the glycoproteins is generally described in Ray et al., Virology, 148, pp. 323-36 (1986) and Ray et al., J. Gen. Virol., 68, pp. 409-18, (1987). The disclosure of each of these latter two articles is incorporated by reference in the present application for patent, as if set forth herein in full. Those experienced in the field of the present invention are quite familiar with the techniques for preparing hybridoma cell lines derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation. Such techniques are described, for example, in Douillard, J-Y. and Hoffman, T., Basic Facts About Hybridomas, in: Compendium of Immunology, Vol. II, L. Schwartz (ed.) (1981); Kohler, G. and Milstein, C., Nature 256, 495-497 (1975); European Journal of Immunology, Vol. 6 pp. 511-519 (1986), Koprowski et al., U.S. Patent 4,172,124, and Koprowski et al., U.S. Patent 4,196,265, the disclosures of which are also incorporated by reference herein.

Regarding specific procedures for performing affinity chromatography, a summary of conventional techniques is provided in Goding, J.W., Monoclonal

Antibodies: Principles and Practice, Academic Press, (1983).

Purified HN and F glycoproteins, prepared as described above, are useful vaccine components, whether used individually or together. Perferrably, however, the two components are combined in an appropriate diluent vehicle or carrier, in the required proportions. Ratios of about 4:1 to about 1:1 HN to F may be employed to provide effective protection against infection.

As noted above, the presence of lipid with the glycoprotein in the vaccine appears to have an unexpected beneficiating effect on the stimulation of the recipient's immune response. Although the mechanism underlying the immunogenic effect observed has not been elucidated, it may be that the lipids function as an adjuvant by enhancing the antigenic effect of the glycoproteins. The endogenous lipid present in the viral envelope, when simultaneously extracted with the glycoprotein by the earlier described procedure, is sufficient to evoke an adequate protective level of antibody production. However, if the vaccine is to be prepared from purified, isolated glycoprotein, it may be desirable to add lipid from an external source in order to obtain the same result seen with the unpurified preparation comprising naturally occurring lipid. By preparing the subunit vaccine in this way the original protein-lipid membrance structure is effectively reconstituted. It has been found that addition of the lipid causes spontaneous formation of vesicles, which comprise the two envelope glycoproteins HN and F, and a lipid bi-layer, thus mimicing the product obtained by solubilization of the viral

envelope followed by dialysis. This procedure may be carried out simply by dissolving the lipid in a dialyzable detergent solution containing the glycoprotein, and dialyzing the solution as described previously in the solubilization procedure. In this manner, not only is it possible to prepare vesicles by combining purified protein with exongenous lipid, but it is also possible, by addition of lipid to the solubilized protein-lipid preparation, to amplify the effect of the endogenous lipid by increasing the natural lipid: protein ratio. Virtually any source of lipid is acceptable for the reconstitution of the vesicular product. Among the lipids contemplated as useful in the present vaccine are phospholipids, representative examples of which are lecithin, cephalin and sphingomycetin. Particularly preferred is lecithin, expecially egg lecithin, a phosphatidly choline.

The subunit vaccine described above may be formulated for intranasal administration with a pharmaceutically acceptable carrier such as water, buffered saline, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) suitable mixtures thereof, or vegetable oils. If necessary, the action of contaminating microorganisms may be prevented by various antibacterial and antifungal agents, such as parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. It will often be preferable to include in the formulation isotonic agents, for example, glucose or sodium chloride. Such formulation may be administered intranasally as an aerosol or atomized spray, or as liquid drops.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like which may be appropriate for intranasal administration of the viral glycoprotein subunit vaccine. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the composition, if neccesary or desirable.

It is especially advantageous to formulate the vaccine in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to a physically discrete unit of vaccine appropriate for the subject to be immunized.. Each dosage should contain the quantity of active material calculated to produce the desired therapeutic effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate vaccine dosage for a given class of recipient are well known to those skilled in art. Generally, when adminstering a composition comprising the HN and F antigens of the virus, a dosage of about 10-200μg should be satisfactory for producing the desired immune response.

The glycoprotein-lipid containing viruses are responsible for causing infections in a wide variety of vertebrate hosts, and the above-described subunit vaccine formulations are adaptable for intranasal adminsitration to any vertebrate host which is

susceptible to these infections. However, the preferred vaccines of the invention, intended for prevention of parainfluenza infection, are most valuable in treatment of mammalian hosts, including man.

The following examples are provided to describe the invention in further detail. These examples are intended to illustrate and not to limit the invention.

Example 1 - Preparation of Vaccine From Human Parainfluenza Type 3 (PI3) Virus

Vaccine was prepared from cultured LLC-MK_2 cells (rhesus monkey kidney) according to methods previously described in Ray et al., J. Infect. Dis. supra, at 1220-21, with protein estimation determined in the same manner as there indicated. Approximately one-third of the total virus protein could be recovered in the detergent soluble fraction. This material showed a significant HA titer (1:320).

Example 2 - Immunization of Test Animals

A - Effect of Dosage on Protection

Three groups of animals (I, II and III) were immunized intranasally at weekly intervals over four consecutive weeks with different doses of the vaccine prepared as described in Example 1, above. The desired quantity of the vaccine in a 100μl volume was slowly instilled in aliquots through both nostils. The tongues of the animals were restrained to minimize swallowing of the vaccine until administration was completed. A parallel group of animals was included as an unimmunized control (IV). Twenty-one days after the last immunization, the animals were challenged intranasally with 10^5 p.f.u. live virus in 100μl. Infected hamsters were sacrificed at 70 hours after infection and blood was collected for serum preparation.

Bronchial lavages from each hamster were collected by slowly instilling and aspirating 1 ml. of phosphate buffered saline (PBS) with a syringe and 18 gauge needle through the trachea. Bronchial lavages were clarified by centrifugation and stored frozen in aliquots. Trachea and lungs of the animals were asceptically removed, suspended in 2 ml of Dulbecco's medium containing 1% BSA lyrine serum albumin and stored frozen until used.

Plaque assays of the hamster lung homogenates were done in accordance with the procedure described in Ray et al., J. Infect. Dis., supra at 1220. No virus was recovered from the lungs of the intranasally immunized test animals after challenge infection.

By contrast, the unimmunized group (IV) of test animals showed virus recovery on the order of 10^4 p.f.u./gm. of tissue, as previously reported. Id. at 1226-27. The results of the just described immunization test are set forth in Table 1A.

B - Effect of Mode of Administration On Protection

In order to further determine the protective immune response using relatively low quantities of the vaccine and to compare the efficacy of intranasal versus subcutaneous administration, another test was conducted with four more groups of hamsters. The test animals in groups V and VI were immunized four times at weekly intervals with 5μg. of the vaccine prepared as in Example 1, above, either through the subcutaneous or intranasal routes. The test animals in group VII were immunized intranasally only three time with 5μg. doses of the vaccine. An unimmunized group of test animals (VIII) was included as a control. This test established that the animals immunized subcutaneously were only partially protected from challenge infection. Virus titers in the lungs of the group V test animals were 100 fold lower than the unimmunized control. On the other hand, using the same quantity of vaccine the animals immunized intranasally showed complete protection from challenge infection. The test animals in group VII also showed only partial protection. The results of this test are set forth in Table 1B.

TABLE 1

Immunization schedule and virus recovery following challenge infection of hamsters with live PI3 virus

Group No.*	Route of Administration	Immunization doses at weekly intervals (in micrograms) Week 0	1	2	3	Virus recovery (pfu/gm) from lungs after challenge infection+
A						
I	Intranasal	5	5	5	5	<10
II	Intranasal	10	10	10	10	<10
III	Intranasal	20	20	20	20	<10
IV	Unimmunized	-	-	-	-	4.7×10^4
B						
V	Subcutaneous	5	5	5	5	1.9×10^4
VI	Intranasal	5	5	5	5	<10
VII	Intranasal	5	5	5	5	1.2×10^2
VIII	Unimmunized	-	-	-	-	4.0×10^4

*Each group consisted of 4 hamsters.

+Virus recovery expressed as the geometric mean titers from four animals.

Example 3 - Determination of Local Immune Response

Bronchial lavages were collected after sacrificing the infected hamsters and used in plaque neutralization tests of the PI3 virus on Vero cell monolayers. The plaque nuetralization tests were conducted according to the prodedure described in Ray et al., J. Infect. Dis., supra, at 1221-22. The test results, which are expressed as the reciprocal of the hightest dilution of serum that inhibits plaque formation by 50%, are set foth in Table 2. These results show that animals immunized subcutaneously four times with 5μg, or intranasally three times with 5μg. of the vaccine prepared as in Example 1, above had a two-fold variation in neutralization titers and partial protection from challenge infection. By contrast, animals immunized intranasally four times with 5μg, 10 μg or 20μg were found to exhibit reciprocal neutralization titers of 20 or higher in their bronchial lavages and were completely resistant to challenge infection.

Sera and bronchial lavages of the test animals were also tested for anti-HN antibodies by HI assay, according to the porcedure set forth in Ray et al., J. Infect. Dis., supra at 1221. The results of these tests are also shown in Table 2. Serum from subcutaneously immunized animals showed a reciprocal titer of 16, whereas bronchial lavages were found to be devoid of HI activity. However, HI activity was detected both in sera and bronchial lavages of animals immunized intranasally with high quantities of

glycoproteins (four times with 10μg. or 20μg.).

Bronchial lavages were also analyzed by immune precipitation to detect the specificity of local antibody for viral polypeptides. In carrying out this analysis, LLC-MK_2 cells were infected with PI3 virus and the infected cells were labeled at 30 hours after infection with ^{35}S-methionine for 3 hours. Cells were lysed with lysis buffer, centrifuged at 13,000 g. for 5 minutes and the clear lysate was used as the source of viral polypeptides. Bronchial lavage (100μl) was mixed with the lysate and the immune precipitates were obtained by adding protein A-sepharose CL-4B beads, precoated with goat anti-hamster whole serum. The sepharose beads were extensively washed and analyzed by SDS-PAGE followed by fluorography as generally set forth in Ray et al., J. Infect. Dis, supra, at 1222.

The results of the immune precipitation anaylysis with representative specimens from each group of hamsters are shown in FIG 3. The ^{35}S-methionine labeled polypeptide profile of PI3 virus appears in lane 1 and that of immune precipitates with bronchial lavages from hamsters in groups I and VI appears in lanes 2 to 7, respectively, which were developed on 10% SDS PAGE. Polypeptides of vesicular stomatitis virus were run in lane 8 as molecular weight markers.

The bronchial lavages of hamsters immunized intranasally with different doses of glycoproteins could effectively precipitate both HN and F polypeptides (lanes 4, 5, 6 and 7) and the relative intesity of HN (68K) appeared to be much higher than F_1 (54K). It is difficult to quantitate the antibody

responses to these glycoproteins from these results, as the higher molecular weight bands (>68K) could not be identified and may represent uncleaved fusion protein (Fo) and aggregated homo-or heteropolymers of HN and F. Bronchial lavages from subcutaneously immunized animals were also found to precipitate both HN and F_1, but with much lower intensitites (lane 3), when compared with the results from intranasally immunized animals. The finding of the nucleocapsid associated protein M polypeptide in immune precipitates with bronchial lavages from animals intranasally immunized with higher quantities of the glycoproteins (lanes 5, 6 and 7) is presumably due to its presence in the vaccine preparation used for immunization.

TABLE 2

NEUTRALIZATION AND
HI TITERS OF BRONCHIAL LAVAGES AND SERA
OF IMMUNIZED AND CONTROL HAMSTERS*

Route of Immunization	Doses of Vaccine	Reciptrocal HI Titer in Bronchial Lavage	Reciprocal Serum HI titer	Reciprocal Neutralization Titers
Intranasal	5 ug x 3	4	<4	10
Intranasal	5 ug x 4	8	<4	20
Intranasal	10 ug x 4	8	8	40
Intranasal	20 ug x 5	8	16	40
Subcutaneous	5 ug x 4	4	16	5
Unimmunized	-	<4	<4	<5

*Titers determined by using 8 HAU of the virus and expressed as the mean value from four animals.

Example 4 - Comparison of Local and Systemic Response

In order to further analyze the immune response an effort was made to detect antibodies to HN and F in sera and bronchial lavages by means of enzyme-linked immunosorbent assay (ELISA). The results of these assays are shown in Table 3. Serum and bronchial lavages were assayed separately to determine the specific antibody responses to HN and F. Affinity purified HN and F were used separately to coat ELISA plates. Antigen coated plates were blocked with 1% BSA in borate-saline before addition of the test specimens. Serial two-fold dilutions of serum or bronchial lavage were incubated with antigen coated wells. Rabbit anti-hamster whole serum was used as the second antibody to determine the total Ig response to viral glycoproteins. The IgA class specific antibody response was determined by using rabbit anti-serum to hamster IgA.

The hamster IgA required for preparation of rabbit antisera to hamster IgA was prepared from pooled sera by lectin affinity chromatography using jacalin (Pierce Chemical Co., Rockford, IL). Jacalin, an α-D-galactose-binding lectin, is extracted from jack-fruit seeds and has been observed to bind specifically with human IgA. Immobilized jacaline on agarose beads was packed in a small disposable plastic column (Biorad Laboratories, Richmond, CA) up to a volume of 4 ml. The column was washed with about 5 column volumes of PBS, pH 7.4. Pooled hamster sera (6 ml.) was dialyzed against PBS and slowly recycled four

times through a jacalin column. The column was washed with ten volumes of PBS and the bound protein was eluted with 0.1 M melibose (Sigma Chemical Co., St. Louis, MO) in PBS and fraction were monitored for absorbance at 280 mm. Eluted fractions were combined and concentrated in a collodion bag (Schleicher and Schuell, Keene, NH).

Hamster serum protein bound to the jacalin column was eluted as a sharp peak with melibiose FIG. 1. Rabbit antiserum raised against the purified protein showed one strong precipitin line in an immunodiffusion test. An additional weak precipitin band was also observed due to the presence of contaminating serum proteins eluted from the jacalin column (not shown). Further analysis by immunoelectrophoresis (IEP) demonstrated that the rabbit antiserum cross-reacted with hamster IgG (H and L-chain specific). This cross-reactivity was eliminated by repeated adsorption of the rabbit antiserum through a Sepharose 4B-hamster IgG column and monitored by IEP (FIG. 2) and ELISA against purified hamster IgG. As appears in FIG. 2, rabbit antiserum to purified IgA (trough 1) and the adsorbed antiserum to hamster IgG (trough 3) were allowed to react with electrophoresed hamster IgG (wells a and c) and hamster whole serum (well b), respectively. Goat antiserum to hamster IgG was used as a control (trough 2). Positions of precipitation arcs which appeared with hamster IgA and IgG are indicated by arrows. The trailing part of the precipitation arc appearing with IgA is probably due to contaminating hamster serum protein present in the IgA preparation from the jacalin column.

The IgA thus obtained was used to raise hyperimmune rabbit antisera. Rabbits were immunized three time intramusclularly each with 100µg. of purified IgA at weekly intervals. The first immunization was offered by emulsifying the protein with Freunds complete adjuvant (Difco Laboratories, Detroit, MI). A second immunization was given similarly with Freund's incomplete adjuvant and the third immunization, with the purifed IgA without adjuvant, was given intramuscularly with a similar quantity of protein. Rabbits were immunized intravenously with another 100µg. of purified IgA and sacrificed by cardiac puncture on the fourth day after the last immunization for preparation and storage of antisera. The antiserum was recycled four times through a column of sepharose-4B coupled with hamster IgG (H & L chain specific) (Southern Biotechnology Associates, Birmingham, AL) to adsorb out is cross-reactivity with hamster IgG. The rabbit antiserum was analyzed by immunodiffusion, immunoelectrophoresis and ELISA to determine its specificity for hamster IgA.

After incubation with second antibody, goat anti-rabbit Ig conjugated wth alkaline phosphatase was added to the wells of the ELISA plates. Finally, p-nitrophenyl phospate was used as the substrate to develop a color reaction and after incubation the reaction was stopped by addition of an equal volume of 2(N) NaOH. Color intensites were measured at 405 nm with a spectrophotometer (Titertek Multiskan ^{R}MC, Flow Laboratories, McLean, VA). Total IgA titers in bronchial wash were measured by coating the plate with jacalin or goat anti-hamster whole serum (Cappel

Philadelphia, PA) and using rabbit anti-hamster IgA as the second antibody. All the ELISA reagents were previously titrated against their counterparts to determine the appropriate dilution to be employed.

According to the results of the ELISA determination, as shown in Table 3, test animals immunized subcutaneously with vaccine preparation as described in Example 1, above, showed a rise in antibody titers in sera but low levels in bronchial lavages. On the other hand, higher antibody responses were observed in bronchial lavages of intranasally immunized hamsters, and the titers increased with an increase in dose of the glycoproteins (groups C, D, E and F). The appearance of glycoprotein specific antibodies as well as IgA class specific responses to both HN and F could be detected in sera and bronchial lavages with intranasally immunized animals. It is interesting to note that intranasal immunization with a higher quantity of vaccine also gives rise to a systemic antibody response, since animals immunized four time with 20µg. had similar Ig and IgA levels of serum antibodies as were observed in subcutaneously immunized group of animals. ELISA titers of antibodies in bronchial lavages were found to be low, which may be due to dilution of the bronchial fluids during their collection.

Further efforts were made to determine the relative proportions of antigen specific IgA and total IgA in bronchial lavages. To determine total IgA titers, bronchial lavages were tested separately with two different reagents, jacalin and goat anti-hamster whole serum, coated on ELISA plates. Similar titers were obtained with both of these reagents. Glyco-

protein specific IgA titers in bronchial lavages were determined separately to affinity purified HN and F and results are shown in FIG. 4, in which optical densities are plotted against dilutions of each bronchial lavage to determine linear portions of the graph for both antigen specific and total IgA titrations. The ratios of the optical densities of antigen specific and total IgA, at a fixed dilution of the two titrations, were multiplied by 100 to represent the relative percentage of antigen specific IgA in bronchial lavage. Anti-HN and anti-F antibody level of animals which were unimmunized (group A), subcutaneously immunized four times with 5μg. (group B), intranasally three times with 5μg. (group C), four times with 5μg. (group D), four times with 10μg. (group E) or four times with 20 μg. (Group F) of vaccine are shown by bar diagram. Upper bars represent the variation within a group of animals.

From FIG. 4 it can be seen that intranasally immunized animals exhibited a significantly higher local IgA response to HN (>15%) and F (>7%) glycoproteins and the anti-HN IgA response was greater than anti-F. Bronchial lavages were also tested against disrupted virus coated on an ELISA plate, and similar IgA responses were also observed. It appeared from a similar experiment that other classes of antigen specific immunoglubins were present in much lower titers.

The test results set forth in the foregoing examples indicate that the above-described glycoprotein subunit vaccine can effectively induce portective immune response in the respiratory tract following administration through the intranasal route. This

appears to be due, at least in part, to induced local antibody production, particularly antibody of the IgA class. The above data further indicate that intranasal immunization requires low quantities of the viral envelope glycoprotein and lipid complex, as compared with subcutaneous administration, in order to confer effective protection from challenge infection.

While certain preferred embodiments of the present invention have been described above, it is not intended to limit the invention to such embodiments, but various modifications may be made thereto, without departing from the scope and spirit of the present invention, as set forth in the following claims.

TABLE 3

ELISA TITERS OF VACCINE-INDUCED SPECIFIC ANTIBODY IN SERUM AND BRONCHIAL LAVAGES OF TEST ANIMALS*

Route of Immunization	Dose of Vaccine	Serum IG Response		Serum IgA Response		Local IG Response		Local IgA Response	
		Anti-HN	Anti-F	Anti-HN	Anti-F	Anti-HN	Anti-F	Anti-HN	Anti-F
Intranasal	5 ug x 3	800	400	400	200	100	50	20	10
Intranasal	5 ug x 4	800	400	400	200	200	100	40	20
Intranasal	10 ug x 4	800	400	400	200	200	100	40	20
Intranasal	20 ug x 4	1,600	800	800	800	400	200	80	80
Subcutaneous	5 ug x 4	3,200	1,600	800	400	50	50	5	5
Unimmunized	-	<100	<100	<100	<100	<50	<50	<5	<5

*Titers expressed as the means reciprocals of highest dilution of samples for four animals showing positive reactivity (O.D. = 0.3).

What is claimed is:

1. A method for immunizing against viral infection comprising administering intranasally an immunogenically effective amount of a viral envelope subunit vaccine comprising a glycoprotein complexed with a lipid.

2. A method as claimed in claim 1, wherein immunization is effected by administering a receptor-binding glycoprotein or a fusion glycoprotein, or a combination thereof, complexed with a lipid.

3. A method as claimed in claim 1, wherein immunization is effected by administering an F glycoprotein and an HN glycoprotein complexed with a lipid.

4. A method as claimed in claim 1, wherein immunization is effeced by administering an F glycoprotein and an HN glycoprotein reconstituted into lipid vesicles.

5. A method for immunizing against infection by a virus selected from the group comprising paramyxoviruses, influenza viruses, respiratory syncytial viruses, rabies virus, herpes viruses and human immunodeficiency viruses said method comprising administering intransally an immunogenically effective amount of a viral envelope subunit vaccine derived from said virus, said vaccine comprising a glycoprotein complexed with a lipid.

6. A method as claimed in claim 5, wherein immunization is effected by administering a receptor-binding glycoprotein or a fusion glycoprotein, or a combination thereof, complexed with a lipid.

7. A method as claimed in claim 5, wherein immunization is effected by administering an F glycoprotein and an HN glycoprotein complexed with a lipid.

8. A method as claimed in claim 5, wherein immunization is effected by administering an F glycoprotein and an HN glycoprotein reconstituted into lipid vesicles.

9. A method for immunizing against infection by a parainfluenza virus, said method comprising administering intranasally an immunogenically effective amount of a viral envelope subunit vaccine derived from said virus, said vaccine comprising a glycoprotein complexed with a lipid

10. A method as claimed in claim 9, wherein immunization is effected by administering a receptor-binding glycoprotein or a fusion glycoportein, or a combination thereof, complexed with a lipid.

11. A method as claimed in claim 9, wherein immunization is effected by administering an F glycoprotein and an HN glycoprotein complexed with a lipid.

12. A method a claimed in claim 9, wherein immunization is effected by administering an F glycoprotein and an HN glycoprotein reconstituted into lipid vesicles.

13. A method for immunizing against infection by human parainfluenza type 3 virus, said method comprising administering intranasally an immunogenically effective amount of a viral envelope subunit vaccine derived from said virus, said vaccine comprising a glycoprotein complexed with a lipid.

14. A method as claimed in claim 13, wherein immunization is effected by administering a receptor-binding glycoprotein or a fusion glycoprotein, or a combination thereof, complexed with a lipid.

15. A method as claimed in claim 13, wherein immunization is effected by administering an F glycoprotein and an HN glycoprotein complexed with a lipid.

16. A method as claimed in claim 14, wherein immunization is effected by administering an F glycoprotein and an HN glycoprotein reconstituted into lipid vesicles.

17. A method as claimed in claim 1 wherein said viral envelope subunit vaccine is produced by genetic engineering.

18. The use of a viral envelope subunit vaccine comprising a glycoprotein complexed with a lipid for the manufacture of a medicament for intranasal administration for immunizing against viral infection.

19. The use as claimed in claim 18, wherein a vaccine as defined in any of claims 2 to 17 is employed.

20. A composition adapted for intranasal administration in a method of immunizing against viral infection, which comprises an immunogenically effective amount of a viral envelope subunit vaccine, comprising a glycoprotein complexed with a lipid, and a therapeutically acceptable carrier suitable for intranasal administration.

21. A composition as claimed in claim 20, wherein a vaccine as defined in any of claims 2 to 17 is used.

22. A composition as claimed in claim 20, in a form administrable as an aerosol or atomized spray, or as liquid drops.

23. A composition as claimed in claim 20 in unit dosage from comprising 10-200 micrograms of said vaccine.

24. A method of preparing a vaccine for immunizing against viral infection, which comprises

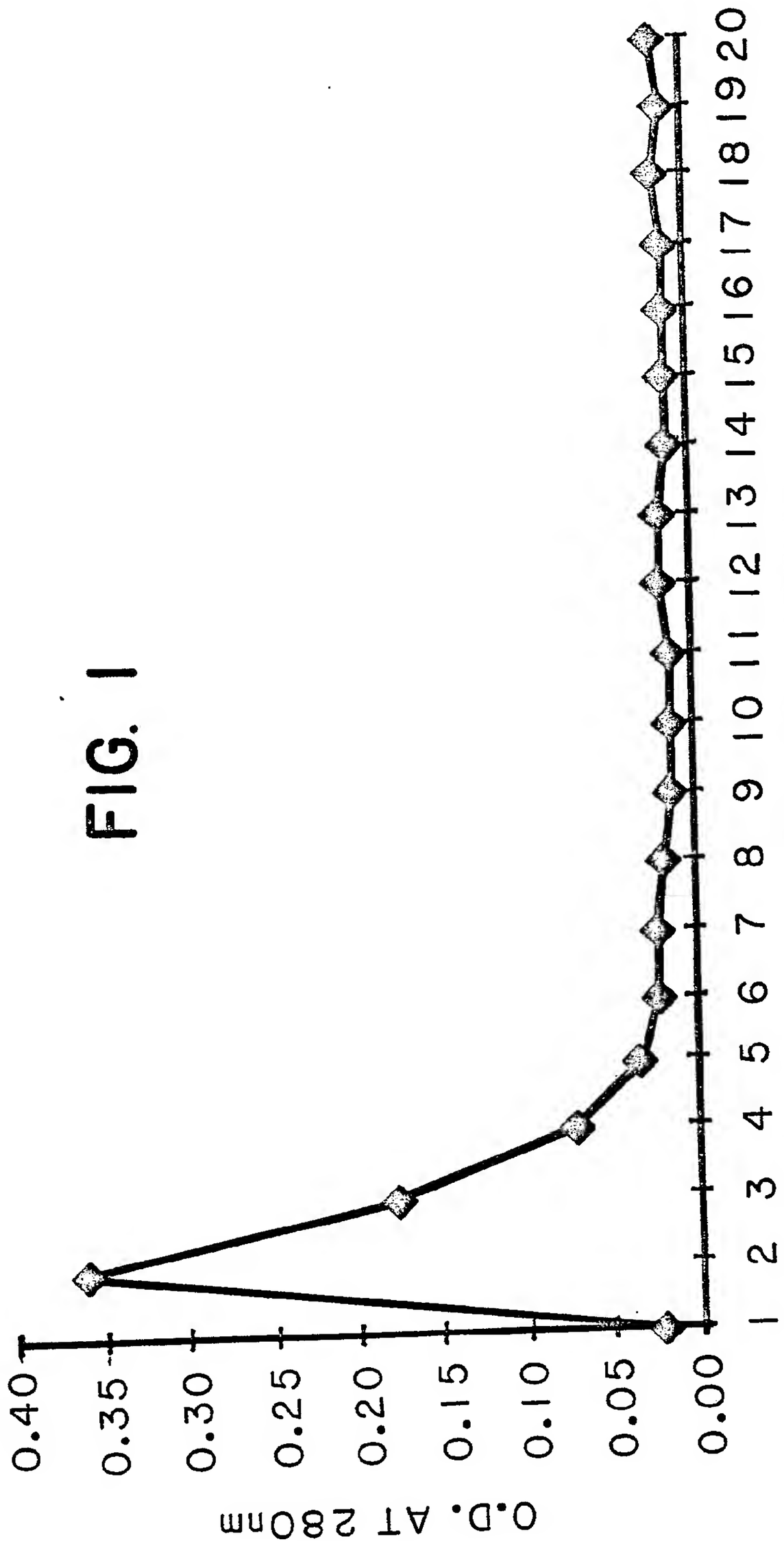
combining (1) an immunogenically effective amount of a viral envelope subunit vaccine comprising a glycoprotein complexed with a lipid, and (2) a pharmaceutically acceptable carrier adapted for intranasal administration.

25. A method as claimed in claim 24, wherein a vaccine as defined in any of claims 2 to 17 is prepared.

26. A method as claimed in claim 24, wherein the vaccine is formulated for administration as an aerosol or atomized spray, or as liquid drops.

27. A method as claimed in claim 24, wherein the vaccine is formulated for an intranasally administrable dosage of 10 - 200 micrograms.

FIG. 1


0.D. AT 280nm
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0.35
0.30
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0.10
0.05
0.00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
FRACTION NUMBER

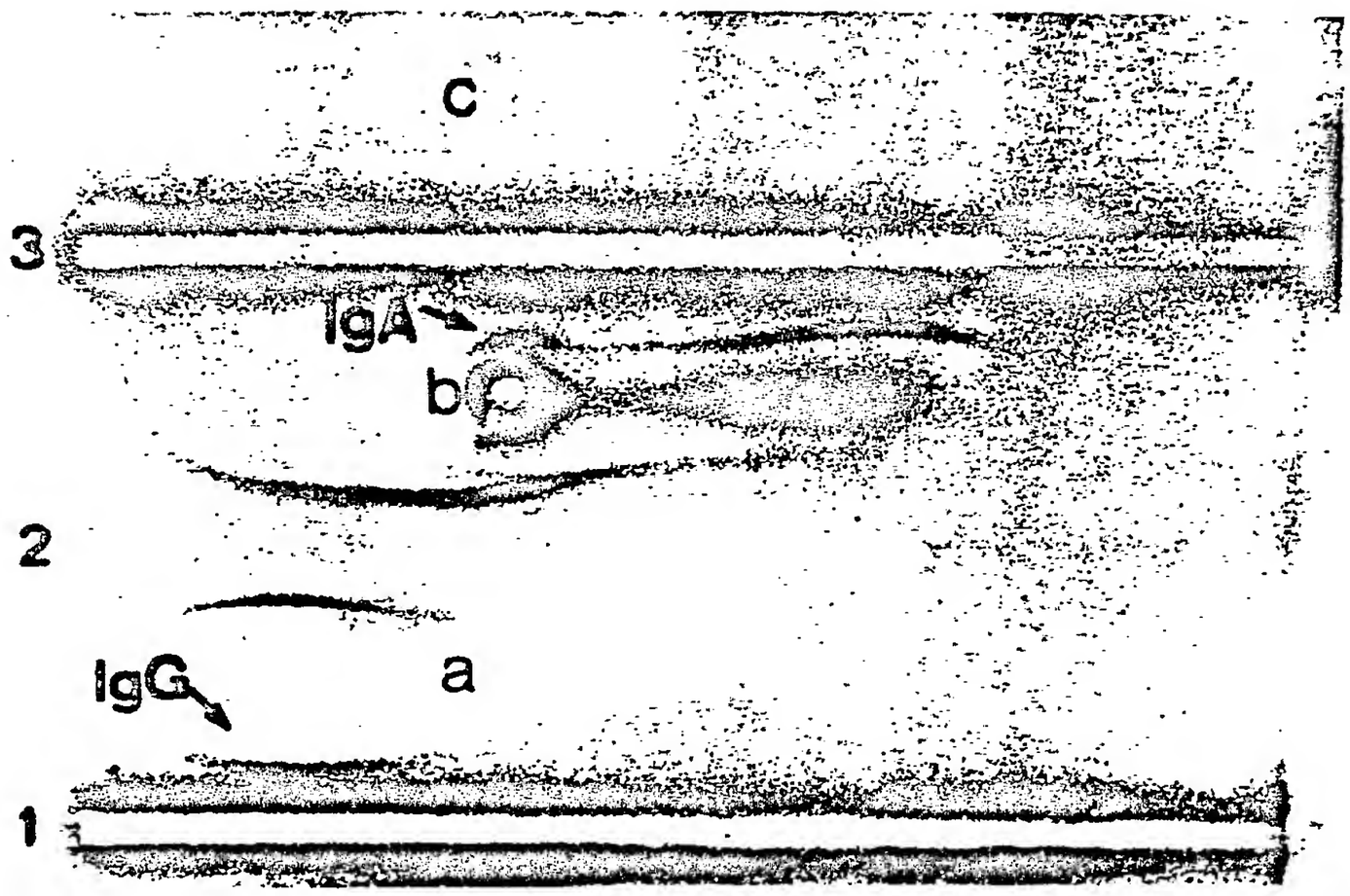


FIG. 2

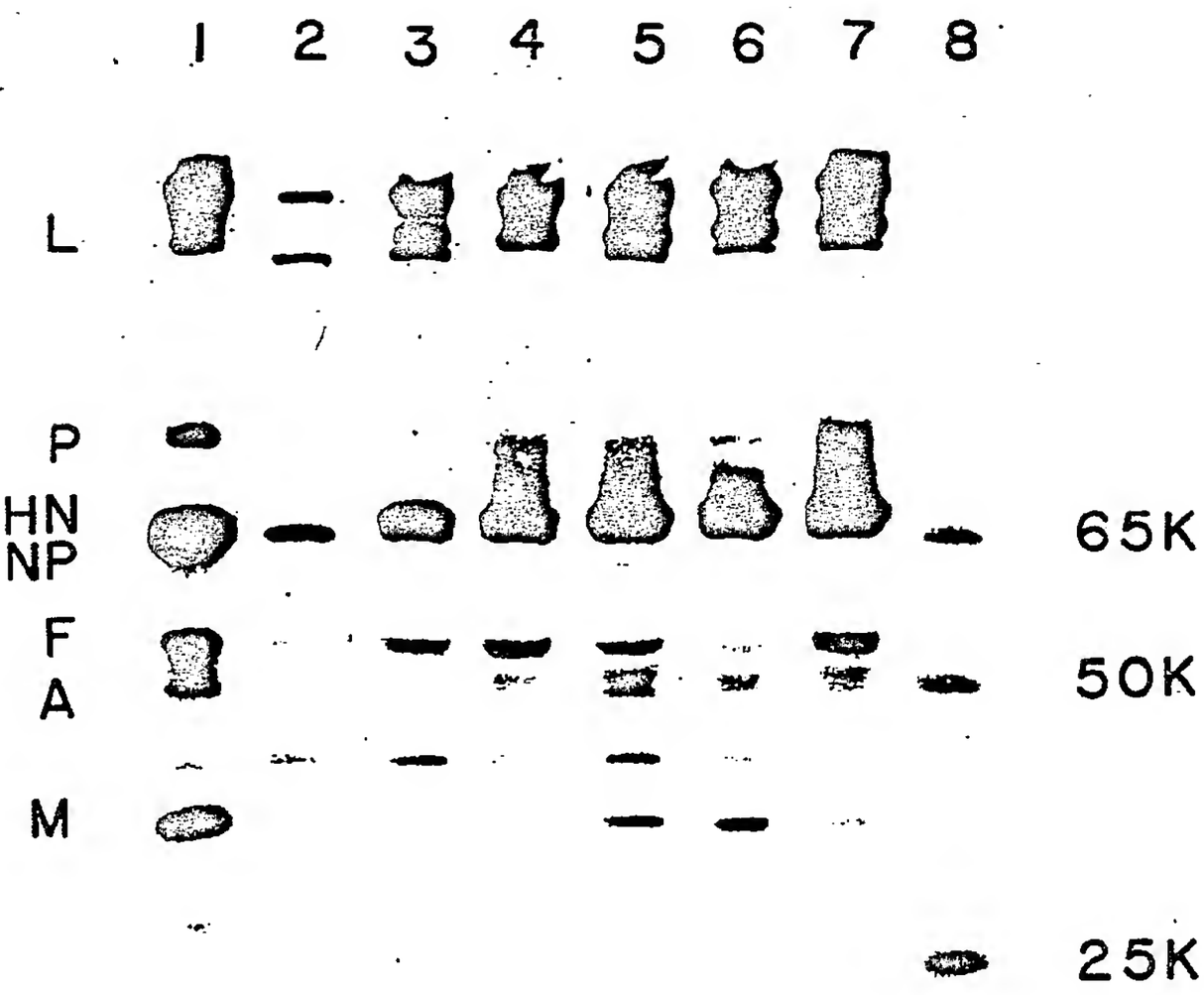
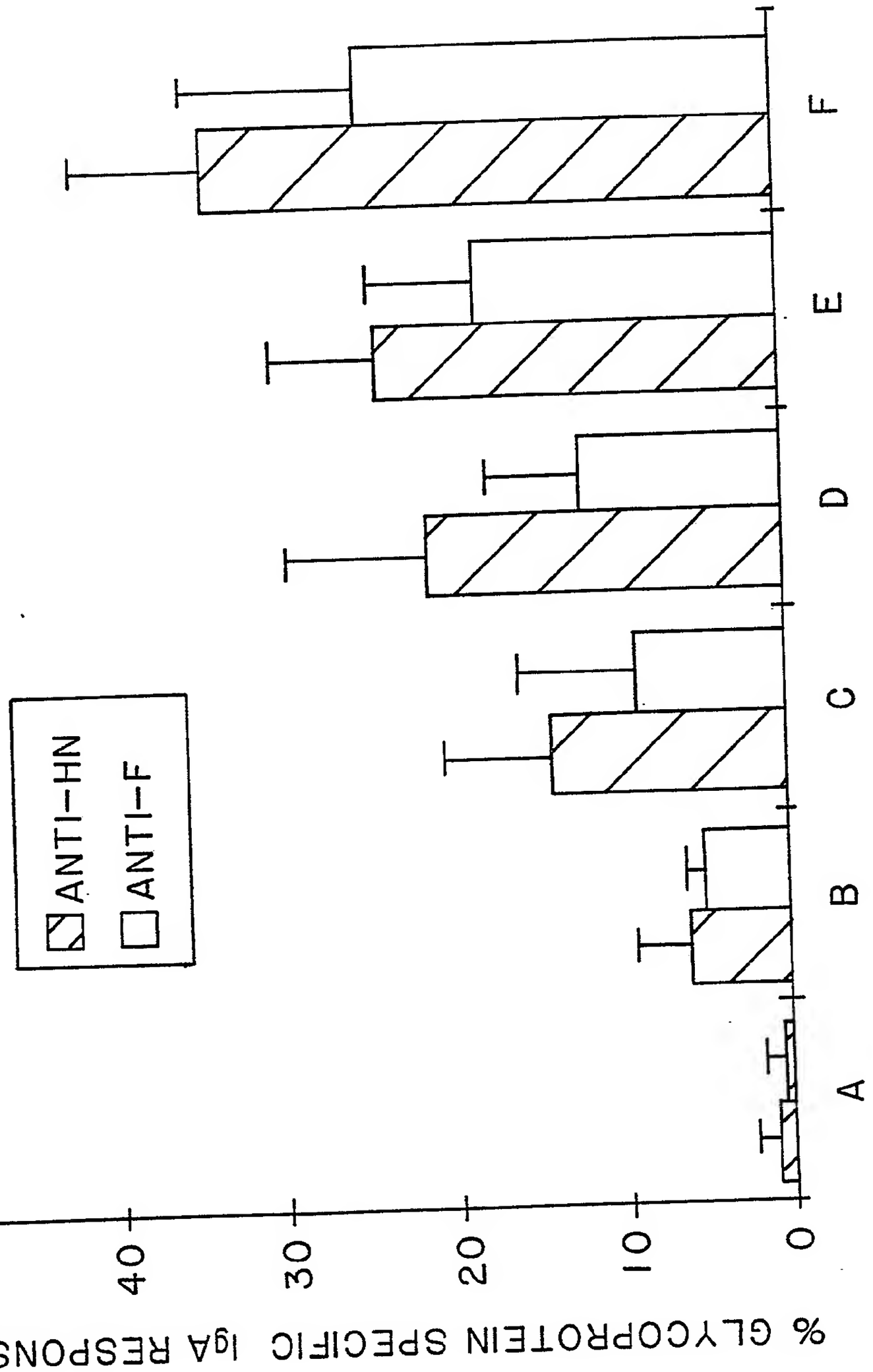


FIG. 3

FIG. 4


ANTI-HN
ANTI-F
% GLYCOPROTEIN SPECIFIC IgA RESPONSE
50
40
30
20
10
0
A
B
C
D
E
F

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01502

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT CL: (4): A61K 39/155, 39/245
U.S. CL. : 424/89

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/89,88; 530/395

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A, 3,544,680, 01 December 1970, (PLOTKIN, ET AL), See the entire document.	1-27
A	US, A, 3,634,587, 11 January 1972, (AMENT ET AL), See the entire document.	1-27
A	US, A, 3,927,208, 16 December 1975, (ZYGRAICH ET AL), See the entire document.	1-27
A	US, A, 3,950,512, 13 April 1976, (EMERY ET AL), See the entire document.	1-27
A	US, A, 3,953,592, 27 April 1976, (PEETERMANS), See the entire document.	1-27
A	US, A, 3,962,422, 08 June 1976, (PARKS), See the entire document.	1-27

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21 July 1988	08 SEP 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/USA	T.D. WESSENDORF

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	US, A, 3,962,421, 08 June 1976, (NEURATH), See the entire document.	1-27
A	US, A, 3,962,423, 08 June 1976, (PEETERMANS ET AL), See the entire document.	1-27
A	US, A, 3,962,424, 08 June 1976, (ZYGRAICH ET AL), See the entire document.	1-27

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4,110,433, 29 August 1978, (PURDY, III), See the entire document.	1-27
A	US, A, 4,132,775, 02 January 1979, (VOLENEC ET AL), See the entire document.	1-27
A	US, A, 4,235,871 25 November 1980, (PAPAHADJOPOULOS ET AL), See the entire document.	1-27
A	US, A, 4,235,877, 25 November 1980, (FULLERTON), See the entire document.	1-27
A	US, A, 4,261,975, 14 April 1981, (FULLERTON ET AL), See the entire document.	1-27
A	US, A, 4,448,765, 15 May 1984, (ASH ET AL), See the entire document.	1-27
A	US, A, 4,512,972, 23 April 1985, (SCHMIDT-RUPPIN), See the entire document.	1-27
Y	US, A, 4,663,161, 05 May 1987, (MANNINO ET AL), See the entire document.	1,2,5,6, 9,10,13, 14,17-21,24 and 25
Y	CA, A,1,158,978, (ADAMOWICZ ET AL) 20 December 1983, See the entire document.	1,2,5,6 9,10,13, 14,17, 21,24 and 25
Y	The Journal of Infections Diseases, Vol. 152, No. 6, issued December 1985, R. Ray, "Glycoproteins of Human Parainfluenza Virus Type 3:Characterization and Evaluation as a Subunit Vaccine", see pages 1219-1230.	1,2,5,6,10, 9,13,14, 17,21,24 and 25
A	Virology, 148, issued 1986, R. Ray, "Monoclonal Antibodies Reveal Extensive Antigenic Differences between the Hemagglutinin Neuraminidase Glycoproteins of Human and Bovine Parainfluenza 3 Viruses", see pages 232-236.	1-27